DESCRIPTION

MATERIALS AND METHODS FOR DETECTION OF *OXALOBACTER FORMIGENES*

This invention was made with government support under National Institutes of Health Grant No. DK 20586. The government has certain rights in this invention.

Cross-Reference to a Related Applications

This application is a continuation of U.S. application Serial No. 09/829,094, filed April 9, 2001, which is a continuation of U.S. application Serial No. 08/936,094, filed September 23, 1997, now U.S. Patent No. 6,214,980; which is a continuation-in-part of co-pending patent application Serial No. 08/883,610, filed June 26, 1997, now U.S. Patent No. 6,090,628; which is a continuation-in-part of co-pending patent application Serial No. 08/717,587, filed September 27, 1996, now U.S. Patent No. 5,912,125; which is a continuation-in-part of co-pending patent application Serial No. 08/493,197, filed June 20, 1995, now U.S. Patent No. 5,837,833; which is a continuation-in-part of co-pending patent application Serial No. 08/262,424, filed June 20, 1994, now U.S. Patent No. 5,604,111.

Field of Invention

The present invention relates to novel assay methods and devices for determining the presence or concentration of oxalate in a sample; *Oxalobacter* genes encoding enzymes required for the catabolism of oxalate; and materials and methods for detecting and identifying *Oxalobacter formigenes* in a sample.

Background of the Invention

Oxalic acid (Oxalate) is a highly toxic natural by-product of catabolism in vertebrate animals and many consumable plants. Unfortunately, a significant portion of humans are unable to properly metabolize oxalate, a condition which may result in the formation of kidney stones in those persons. It is estimated that 70% of all kidney stones are composed of some amount of oxalate. Approximately 12 percent of the U.S. population will suffer from a kidney stone at some time in their lives, and the incidence is rising not only in the United States, but also in Sweden and Japan (Curhan, 1993). Moreover, although a healthy person breaks down or excretes sufficient quantities of oxalate to avoid excessive accumulation of oxalate in the tissues, a number of disease states are known to be associated with malfunctions of oxalate metabolism, including pyridoxine deficiency, renal failure and primary hyperoxaluria, a metabolic genetic disorder that results in the excessive deposition of oxalate in the kidneys.

Persons suffering from and at risk for developing kidney stones, as well as patients with lipid malabsorption problems (*e.g.*, sprue, pancreatic insufficiency, inflammatory intestinal disease, bowel resection, etc.), tend to have elevated levels of urinary oxalate, a fact that has been exploited as a means for identifying individuals at risk. While elevated levels of oxalate may be present in urine, detecting elevated levels of oxalate in serum has not been routine due to the difficulty in detecting the low levels of oxalate present in serum.

Most previous methods for measuring oxalate in a biological sample first require the isolation of the oxalate by precipitation, solvent extraction, or an ion-exchange absorption (Hodgkinson, 1970). Quantitation of the isolated oxalate may be determined by any one of several methods including colorimetry, fluorometry, gas-liquid chromatography or isotope dilution techniques. Because many of the oxalate isolation techniques used in these analytical methods are not quantitative, it is normally necessary to correct for the low recovery of oxalate by adding a ^{14}C-labeled oxalic acid internal standard, which further complicates the analytical method. All these methods are laborious, and consequently expensive because of

the amount of skilled laboratory technician time which must be employed. In addition, isolation of the oxalate may require relatively large sample volumes for starting material.

Recently, several advances in the detection and quantitation of oxalate have been made through the use of (a) oxalate degrading enzymes and (b) high performance liquid chromatography. One commercially-available enzymatic test (Sigma Chemical Company, St. Louis, MO) employs oxalate oxidase to oxidize oxalate to carbon dioxide and hydrogen peroxide. The hydrogen peroxide produced can then be measured colorimetrically in a second enzymatic reaction in the presence of peroxidase.

In another enzymatic method for measuring oxalate, oxalate decarboxylase is used to convert oxalate to carbon dioxide and formate. The resultant carbon dioxide can be measured manometrically, by the pH change in a carbon dioxide trapping buffer or by the color change in a pH indicator buffer. Whatever method of carbon dioxide assay is adopted, the time required for diffusion and equilibration of carbon dioxide is much longer than is desirable for a rapid analytical method.

Alternatively, the formate produced by the action of oxalate decarboxylase can be assayed with formate dehydrogenase in an NAD/NADH coupled reaction, as described in Costello, 1976 and Yriberri, 1980. This method is both cumbersome and time-consuming because oxalate decarboxylase and formate dehydrogenase differ in their optimum pH requirements, thus necessitating a pH adjustment during the analysis.

Another commercially available enzymatic test (Boehringer Mannheim) cleaves oxalate to formate and carbon dioxide, then oxidizes the formate to bicarbonate by NAD in the presence of the enzyme formate dehydrogenase. The amount of NADH is determined by means of its absorbance at 334, 340, or 365 nm. Another test ("STONE RISK" by Mission Pharmacal) measures oxalate as a part of a battery of tests for kidney stones.

Oxalobacter formigenes is a recently discovered, oxalate-degrading obligately anaerobic bacterium residing primarily in the intestines of vertebrate animals, including man (Allison *et al.*, 1986). Although the first isolates of *O. formigenes* were cultured from sheep

rumen (Dawson *et al.*, 1980), additional strains have now been isolated from fecal contents of rats, guinea pigs and pigs (Argenzio *et al.*, 1988, Daniel *et al.*, 1987), fecal samples from man (Allison *et al.*, 1985), and anaerobic aquatic sediments (Smith *et al.*, 1985). This bacterium is unique among oxalate-degrading organisms having evolved a total dependence on oxalate metabolism for energy (Dawson *et al.*, 1980). Recent evidence suggests that *Oxalobacter formigenes* has an important symbiotic relationship with vertebrate hosts by regulating oxalic acid absorption in the intestine as well as oxalic acid levels in the plasma (Hatch and Freel, 1996). Studies by Jensen and Allison (1994) comparing various *O. formigenes* isolates revealed only limited diversity of their cellular fatty acids, proteins, and nucleic acid fragments. Based on these comparisons, strains of *O. formigenes* have been divided into two major subgroups. In general, group I strains have shown limited intragroup diversity, while group II strains have shown greater intragroup diversity.

Special conditions are required to culture *O. formigenes* and their detection is based generally on the appearance of zones of clearance of calcium oxalate crystals surrounding colonies (Allison *et al.*, 1986). Assays based on the appearance of zones of clearance of calcium-oxalate crystals surrounding bacterial colonies (Allison *et al.*, 1985) or degradation of oxalate in culture media measured by calcium-chloride precipitation (Dawson *et al.*, 1980) fail to confirm the oxalate-degrading bacteria as *Oxalobacter*.

As illustrated above, the currently existing assays for oxalate suffer from numerous problems, including cost, inaccuracy, reliability, complexity, and lack of sensitivity. Accordingly, it is an object of the subject invention to provide a simple, accurate, and sensitive assay for the detection of low levels of oxalate in a biological sample.

The current methods for culturing and identifying the presence of *Oxalobacter formigenes* are technically demanding and time consuming, and therefore, are not suitable for rapid and specific identification of *O. formigenes*, particularly for clinical diagnostics. Accordingly, another object of the subject invention is to provide a rapid, accurate polynucleotide probe-based assay for the detection of *O. formigenes*.

Brief Summary of the Invention

The subject invention concerns the cloning, sequencing, and expression of the formyl-CoA transferase (*frc*) and the oxalyl-CoA decarboxylase (*oxc*) genes of *Oxalobacter formigenes*, and the use of the enzymes to detect the presence of oxalate in a sample. The assay of the subject invention provides, for the first time, a rapid, sensitive method to detect even very low concentrations of oxalate in biological samples. Advantageously, the biological samples in which oxalate can be detected include both urine and serum samples. The enzyme system used according to the subject invention converts oxalate to carbon dioxide and formate. In a preferred embodiment of the subject invention, the production of formate is then measured colorimetrically. This assay provides a sensitive, accurate and convenient means for detecting oxalate.

A further aspect of the subject invention is the discovery of the *O. formigenes* genes which encode the formyl-CoA transferase and the oxalyl-CoA decarboxylase enzymes. The discovery of these genes makes it possible to efficiently produce large quantities of pure formyl-CoA transferase and oxalyl-CoA decarboxylase for use in the assay of the subject invention or other appropriate application.

The subject invention further concerns a dipstick device for the detection and quantitation of oxalate in a sample. The dipstick device comprises the oxalyl-CoA decarboxylase and formyl-CoA transferase enzymes of the present invention immobilized on a carrier matrix. A detectable signal is generated on the dipstick if oxalate is present in the sample.

The subject invention also provides a means for detecting the presence of *Oxalobacter formigenes* organisms in a sample. The method of detection provided for herein involves polynucleotide probes which can be used to identify *Oxalobacter formigenes*.

The subject invention also concerns the polynucleotide primers and the use thereof for polymerase chain reaction (PCR) amplification of *Oxalobacter formigenes* nucleotide

sequences. Amplified *Oxalobacter* sequences can then be detected using the polynucleotide probes of the subject invention.

Brief Description of the Drawings

Figures 1A-1E show the detection of varying concentrations of oxalate in a sample. Colorimetric absorbance for each sample was plotted over time (minutes). Positive and negative control panels are also shown.

Figures 2A-2B show the nucleotide sequence of the formyl-CoA transferase gene and the deduced amino acid sequence of the formyl-CoA transferase polypeptide from *Oxalobacter formigenes*. Bolded letters represent amino acid residues determined by N-terminal protein sequencing.

Figures 3A-3B show the nucleotide sequence of the oxalyl-CoA decarboxylase gene and flanking regions from *Oxalobacter formigenes*. The consensus ribosome-binding site lies approximately 10 bases upstream (double-underlined letters) from the putative translation initiation codon (positions 1 to 3). A rho-independent termination sequence lies at positions 1758 to 1790 (double-underlined letters). A putative TPP-binding site appears between positions 1351 and 1437.

Figure 4 shows an RFLP analysis of *O. formigenes, strain OxB* using probes specific for the *oxc* gene encoding oxalyl-CoA decarboxylase and the *frc* gene encoding formyl-CoA transferase. Genomic DNA isolated from a 14 day culture of *O. formigenes strain OxB* was digested with the restriction enzyme HIND III. The digested DNA was size fractionated by electrophoreses through 0.5% agarose gels, electroblotted to a nylon membrane, then hybridized with either probe AP15 (SEQ ID NO. 6) or probe AP34 (SEQ ID NO. 9) to detect *oxc* or probe AP273 (SEQ ID NO. 10) to detect *frc*.

Figure 5 shows the sensitivity of detecting the *oxc* and *frc* genes in RFLP of *O. formigenes strain OxB* versus *strain HC-1*. Genomic DNA from each of the two strains was digested with the restriction enzyme HIND III. Two-fold serial dilutions were made of the

digested DNA and size fractionated by electrophoresis through 0.5% agarose gels (left panels). RFLP analyses were carried out as described in Fig. 4, except the nylon membranes were hybridized with a 1:1 mixture of probe AP15 (SEQ ID NO. 6) plus probe AP273 (SEQ ID NO. 10) (right panels)..

Figure 6 shows the detection of the *oxc* and *frc* genes in various strains of *O. formigenes* by RFLP analysis. RFLP was carried out as described in Fig. 5.

Figure 7 shows PCR-based amplification of a genetic region of the *oxc* gene in various strains of *O. formigenes*. Using PCR primer AP15 (SEQ ID NO. 6) and primer AP22 (SEQ ID NO. 11) as PCR primers, PCR amplification was performed using genomic DNA isolated from each of the 12 strains of *O. formigenes* listed in Table 1 as template. PCR products were size fractionated by electrophoresis through 1.2% agarose gels and observed visually using ethidium bromide (EtBr) and UV light.

Figure 8 shows a direct analysis of fecal samples for *O. formigenes*. *Oxalobacter* negative stool sample (A & B) was spiked with 10^2 (C) and 10^4 (D) cfu of OxB or 10^3 (E) and 10^4 (F) cfu of OxK per 0.1 gm. DNA from an unspiked *O. formigenes*-positive stool sample diluted 1:25 (G) and 1:50 (H).

Figures 9A-9B show the identification of sequence homologies within the *oxc* gene expressed in representative group I and group II strains of *Oxalobacter formigenes* to design oligonucleotide probes. Partial sequences of 5'-end of the *oxc* gene generated by PRC amplification of the region bounded by the primer pair, AP34/AP21. A region of high homology shared by all strains (between bp 13 and 43) was selected for the genus-specific oligonucleotide probe, AP286, while regions of high homology shared by only group I strains (between bp 197 and 214) or shared only by group II strains (between bp 133 and 150) were selected for group-specific oligonucleotide probes, HS2 and AP307, respectively.

Figures 10A-10B show the detection of *Oxalobacter formigenes* using a genus-specific oligonucleotide probe that hybridizes to the PCR product of the *oxc* gene. Using the primer pair AP34/AP21, PCR amplification was performed using genomic template DNA

isolated from 8 group I and 8 group II strains of *O. formigenes*. The PCR products were size fractionated by electrophoresis through 1.2% agarose gels and the expected 504-508 bp product visualized with EtBr under UV light (upper panel). The PCR products were transblotted to nylon membranes and Southern blotted using the genus -specific oligonucleotide probe, AP286 (lower panel).

Figures 11A-11C show the classification of group I and group II strains of *Oxalobacter formigenes* using group-specific oligonucleotide probes that hybridize with PCR products of the *oxc* gene. Using the primer pair AP34/AP21, PCR amplification was performed using genomic template DNA isolated from 8 group I and 8 group II strains of *O. formigenes*. The PCR products were size fractionated by electrophoresis through 1.2% agarose gels and the expected 504-508 bp product visualized with EtBr under UV light (upper panel). The PCR products were transblotted to nylon membranes and Southern blotted using HS2, the group I-specific (center panel), or AP307, the group II-specific (lower panel), oligonucleotide probes.

Figure 12 shows agarose gels containing *oxc* amplification products from quantitative PCR visualized with UV light (Figures 12A-12C). Figures 12D-12E show Log equivalence plots to determine the number of *oxc* molecules in the sample.

Brief Description of the Sequences

SEQ ID NO. 1 is a nucleotide sequence for the formyl-CoA transferase gene (also shown in Figure 2).

SEQ ID NO. 2 is a polypeptide encoded by SEQ ID NO. 1, which can be used according to the subject invention.

SEQ ID NO. 3 is the nucleotide sequence for the oxalyl-CoA decarboxylase gene (also shown in Figure 3).

SEQ ID NO. 4 is a polypeptide encoded by SEQ ID NO. 3, which can be used according to the subject invention.

SEQ ID NO. 5 is an oxalyl-CoA decarboxylase sequence, which can be used as a probe according to the subject invention.

SEQ ID NO. 6 is an oxalyl-CoA decarboxylase sequence, which can be used as a probe or PCR primer according to the subject invention.

SEQ ID NO. 7 is an oxalyl-CoA decarboxylase 5′-primer, which can be used according to the subject invention.

SEQ ID NO. 8 is an oxalyl-CoA decarboxylase 3′-primer, which can be used according to the subject invention.

SEQ ID NO. 9 is an oxalyl-CoA decarboxylase sequence, which can be used as a probe or primer according to the subject invention.

SEQ ID NO. 10 is a formyl-CoA transferase sequence, which can be used as a probe according to the subject invention.

SEQ ID NO. 11 is an oxalyl-CoA decarboxylase sequence, which can be used as a PCR primer according to the subject invention.

SEQ ID NO. 12 is an oxalyl-CoA decarboxylase sequence, which can be used as a PCR primer according to the subject invention.

SEQ ID NO. 13 is an oxalyl-CoA decarboxylase sequence, which can be used as a PCR primer according to the subject invention.

SEQ ID NO. 14 is an oxalyl-CoA decarboxylase sequence, which can be used as a probe according to the subject invention.

SEQ ID NO. 15 is an oxalyl-CoA decarboxylase sequence, which can be used as a probe according to the subject invention.

SEQ ID NO. 16 is an oxalyl-CoA decarboxylase sequence, which can be used as a probe according to the subject invention.

SEQ ID NO. 17 is an oxalyl-CoA decarboxylase sequence, which can be used as a PCR primer according to the subject invention.

SEQ ID NO. 18 is a formyl-CoA transferase sequence, which can be used as a PCR primer according to the subject invention.

SEQ ID NO. 19 is a formyl-CoA transferase sequence, which can be used as a PCR primer according to the subject invention.

Detailed Description of the Invention

The subject invention provides an accurate, sensitive assay for oxalate in biological samples such as urine and serum. Elevated levels of oxalate are correlated with urinary tract stone formation, as well as other health problems. Early detection of high levels of oxalate makes it possible to prevent, delay or reduce adverse health consequences through appropriate medication and through modulation of diet.

In the presently described diagnostic system, two enzymes are used to catabolize oxalate to carbon dioxide and formate. Specifically, any oxalate that may be present in a sample being assayed is converted into formate and carbon dioxide (CO_2) through the combined action of the enzymes oxalyl-CoA decarboxylase and formyl-CoA transferase. The formate can then be detected using a variety of techniques known in the art. In a preferred embodiment, the production of formate is measured colorimetrically by linking the catabolism of formate with the production of a detectable color change (for example, the formation of a compound that absorbs a particular wavelength of light). The production of formate is directly correlated with the amount of oxalate present in the sample. Therefore, if a known amount of formate is produced using the subject enzyme system, then the amount of oxalate present in the sample can be easily quantitated.

In a preferred embodiment, the enzymes used in the subject invention are expressed by genes from the bacterium *Oxalobacter formigenes*. The genes encoding both oxalyl-CoA decarboxylase (Lung *et al.*, 1994) and formyl-CoA transferase enzymes have been cloned and expressed, thus providing a readily-available source of reagent material. The subject assay is capable of detecting oxalate levels in a range as low as 0.00025-0.0005 mM (Figures 1A-1E). This level of sensitivity makes the subject assay capable of direct detection of oxalate

in serum samples consisting of little as 10 µl volume. The described system can be easily automated with standard systems known in the art.

In a preferred embodiment of the subject assay, the enzymatic reaction can be carried out in the wells of flat-bottomed 96-well microtiter plates and read in an automated plate reader. Suitable concentrations of the assay reagents oxalyl-CoA decarboxylase, oxalyl-CoA, β-NAD, formate dehydrogenase, and the sample to be assayed are added to the microtiter wells. The reaction is then brought to equilibrium (two minute incubation at 37°C in the plate reader) to permit degradation of any residual formate that may be present in the sample. The formyl-CoA transferase enzyme is then added to the mixture to start the reaction, and the plate is read at 15 second intervals. Formate production is determined by measuring the reduction in NAD in the presence of formate dehydrogenase by detecting changes in absorbance of the sample at 340 nm (Baetz and Allison, 1989). The quantity of oxalate is determined by comparison of the unknown samples with standards having a known amount of oxalate.

Further, the enzymatic reaction of the subject assay will not be initiated until the formyl-CoA transferase, oxalyl-CoA decarboxylase, and oxalyl-CoA are all present within the reaction mixture. Therefore, initiation of the enzymatic reaction can be prevented by withholding one of the above reagents from the reaction mix. Preferably, oxalyl-CoA decarboxylase and oxalyl-CoA are added first, and the reaction is initiated by the addition of formyl-CoA transferase to the mix. However, the order of addition of the three reagents is not material to the function of the assay, so long as one of the reagents is withheld until just prior to the desired initiation point of the assay.

The formyl-CoA transferase and oxalyl-CoA decarboxylase enzymes used in the subject invention can be obtained and purified as a natural product of *Oxalobacter formigenes* (Baetz and Allison, 1989 and 1990). Alternatively, the enzymes can be obtained from host cells expressing the recombinant polynucleotide molecules of the subject invention that encode the enzymes. Other reagents used in the subject assay can be obtained from conventional sources, such as Sigma Chemical Company, St. Louis, MO. Further, a person

of ordinary skill in the art can readily determine the optimal concentrations of the reagents to use in the assay described herein.

A further aspect of the subject invention concerns the cloning, sequencing and expression of the *Oxalobacter formigenes* gene which encodes the formyl-CoA transferase used in the assay that is a subject of the invention. The gene was cloned using degenerate oligonucleotide probes (based on partial amino acid sequencing of tryptic peptides) to screen an *Oxalobacter* genomic DNA library. The gene encodes a polypeptide having a molecular weight of approximately 40 kD. The subject invention further concerns the cloning, sequencing, and expression of the gene which encodes oxalyl-CoA decarboxylase from *Oxalobacter formigenes*. The nucleotide sequence of the cDNA of formyl-CoA transferase and oxalyl-CoA decarboxylase are shown in Figures 2A-2B and 3A-3B, respectively (SEQ ID NOS. 1 and 3).

Because of the redundancy of the genetic code, a variety of different polynucleotide sequences can encode the formyl-CoA transferase polypeptide disclosed herein. It is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, polypeptide of the subject invention. These variant or alternative polynucleotide sequences are within the scope of the subject invention. As used herein, references to "essentially the same" sequence refers to sequences which encode amino acid substitutions, deletions, additions, or insertions which do not materially alter the functional enzymatic activity of the encoded polypeptide. Further, the subject invention contemplates those polynucleotide molecules having sequences which are sufficiently homologous with the DNA sequences shown in Figures 2A-2B and 3A-3B (SEQ ID NOS. 1 and 3) so as to permit hybridization with those sequences under standard high-stringency conditions. Such hybridization conditions are conventional in the art (see, *e.g.*, Maniatis *et al.*, 1989).

As a person skilled in the art would appreciate, certain amino acid substitutions within the amino acid sequence of the polypeptide can be made without altering the functional activity of the enzyme. For example, amino acids may be placed in the following classes:

non-polar, uncharged polar, basic, and acidic. Conservative substitutions, whereby an amino acid of one class is replaced with another amino acid of the same class, fall within the scope of the subject invention so long as the substitution does not materially alter the enzymatic reactivity of the polypeptide. Non-conservative substitutions are also contemplated as long as the substitution does not significantly alter the functional activity of the encoded polypeptide.

The polynucleotides of the subject invention can be used to express the recombinant formyl-CoA transferase enzyme. They can also be used as a probe to detect related enzymes. The polynucleotides can also be used as DNA sizing standards.

The polypeptides encoded by the polynucleotides can be used to raise an immunogenic response to the formyl-CoA transferase enzyme. They can also be used as molecular weight standards, or as inert protein in an assay. The polypeptides can also be used to detect the presence of antibodies immunoreactive with the enzyme.

The polynucleotide sequences of the subject invention may be composed of either RNA or DNA. More preferably, the polynucleotide sequences are composed of DNA. The subject invention also encompasses those polynucleotides that are complementary in sequence to the polynucleotide sequences disclosed herein.

Another aspect of the subject invention pertains to kits for carrying out the enzyme assay for oxalate. In one embodiment, the kit comprises, in packaged combination and in relative quantities to optimize the sensitivity of the described assay method, (a) the oxalyl-CoA decarboxylase, oxalyl-CoA, β-NAD, and formate dehydrogenase; and (b) formyl-CoA transferase. The kit may optionally include other reagents or solutions, such as buffering and stabilization agents, along with any other reagents that may be required for a particular signal generation system. Other reagents such as positive and negative controls can be included in the kit to provide for convenience and standardization of the assay method.

The subject invention further concerns a method for detecting the presence of *Oxalobacter formigenes* organisms in a sample. Specific polynucleotide probes can be prepared based on the nucleotide sequence of either the oxalyl-CoA decarboxylase or the

formyl-CoA transferase gene sequence of *Oxalobacter formigenes*. The polynucleotide probes of the subject invention can be used to identify *Oxalobacter formigenes* in a sample, and to classify the strain of *Oxalobacter formigenes* detected. The polynucleotide probes of the subject invention can be used according to standard procedures and conditions to specifically and selectively detect polynucleotide sequences that have sufficient homology to hybridize with the probe. DNA can be isolated from bacterial microorganisms in a biological specimen (*e.g.*, biopsy, fecal matter, tissue scrapings, *etc.*) using standard techniques known in the art and the isolated DNA screened for hybridization with *Oxalobacter* oxalyl-CoA decarboxylase-specific and/or formyl-CoA transferase-specific polynucleotide probes. Various degrees of stringency can be employed during the hybridization, depending on the amount of probe used for hybridization, the level of complementarity (*i.e.*, homology) between the probe and target DNA fragment to be detected. The degree of stringency can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Hybridization methods and conditions are known in the art and are generally described in *Nucleic Acid Hybridization: A Practical Approach* (Hames, B.D., S.J. Higgins, eds.), IRL Press (1985).

The polynucleotide probes of the subject invention include, for example, the oxalyl-CoA decarboxylase probe A (SEQ ID NO. 5), probe AP15 (SEQ ID NO. 6), and probe AP34 (SEQ ID NO. 9), probe AP 286 (SEQ ID NO. 14), probe AP307 (SEQ ID NO. 15), and probe HS-2 (SEQ ID NO. 16), specifically exemplified herein. Probes for formyl-CoA transferase include, for example, probe AP273 (SEQ ID NO. 10) specifically exemplified herein. The nucleotide sequences of the exemplified probes are shown below:

Probe A 5′- GAGCGATACCGATTGGA -3′ (SEQ ID NO. 5)

Probe AP15 5′- GCACAATGCGACGACGA -3′ (SEQ ID NO. 6)

Probe AP34 5′- ATACTCGGAATTGACGT -3′ (SEQ ID NO. 9)

Probe AP273 5′-TTCATGTCCAGTTCAATCGAACG-3′ (SEQ ID NO. 10)

Probe AP286 5′-GACAATGTAGAGTTGACTGATGGCTTTCATG-3′ (SEQ ID NO. 14)

Probe AP307 5′-CAGGATGGTCAGAAGTTC-3′ (SEQ ID NO. 15)

Probe HS-2 5′-CCGGTTACATCGAAGGA-3′ (SEQ ID NO. 16)

The polynucleotide probes contemplated in the subject invention also include any polynucleotide molecule comprising a nucleotide sequence capable of specifically hybridizing with oxalyl-CoA decarboxylase or formyl-CoA transferase polynucleotide sequence of the present invention. As used herein, reference to "substantial homology" or "substantially complementary" refers not only to polynucleotide probes of the subject invention having 100% homology with the nucleotide sequence of the target polynucleotide, or fragments thereof, but also to those sequences with sufficient homology to hybridize with the target polynucleotide. Preferably, the degree of homology will be 100%; however, the degree of homology required for detectable hybridization will vary in accordance with the level of stringency employed in the hybridization and washes. Thus, probes having less than 100% homology to the oxalyl-CoA decarboxylase or formyl-CoA transferase polynucleotide sequences can be used in the subject method under appropriate conditions of stringency. In a preferred embodiment, high stringency conditions are used. In addition, analogs of nucleosides may be substituted for naturally occurring nucleosides within the polynucleotide probes. Such probes having less than 100% homology or containing nucleoside analogs are within the scope of the subject invention. The skilled artisan, having the benefit of the disclosure contained herein, can readily prepare probes encompassed by the subject invention.

In addition, the subject invention also concerns polynucleotide primers that can be used for polymerase chain reaction (PCR) amplification of *Oxalobacter formigenes* nucleotide sequences. PCR amplification methods are well known in the art and are described in U.S. Patent Nos. 4,683,195; 4,683,202; and 4,800,159. The polynucleotide primers and probes of the present invention can also be used to amplify nucleotide sequences encoding oxalyl-CoA decarboxylase and formyl-CoA transferase by non-PCR based amplification methods. The subject primers and probes in any method where amplification of specific sequences is desired. In a preferred embodiment, the polynucleotide primers are based on the oxalyl-CoA decarboxylase or formyl-CoA transferase gene sequence and can be used to amplify the full

length or a portion of the target gene. The amplified *Oxalobacter* sequences can be detected using the probes of the subject invention according to standard procedures known in the art.

The polynucleotide primers of the subject invention include, for example, oxalyl-CoA decarboxylase PCR primer 1 (SEQ ID NO. 7), PCR primer 2 (SEQ ID NO. 8), PCR primer AP15 (SEQ ID NO. 6), PCR primer AP22 (SEQ ID NO. 11), PCR primer AP34 (SEQ ID NO. 9), PCR primer AP21 (SEQ ID NO. 17), OXF6 (SEQ ID NO. 12), and OXF7 (SEQ ID NO. 13), specifically exemplified herein. The nucleotide sequences of the exemplified PCR primers are shown below:

PCR primer 1	5′- CAGGTTATGCAGCTTCT -3′ (SEQ ID NO. 7)
PCR primer 2	5′- GGATGGTTGTCAGGCAG -3′ (SEQ ID NO. 8)
PCR primer AP15	5′-GCACAATGCGACGACGA-3′ (SEQ ID NO. 6)
PCR primer AP22	5′-GTAGTTCATCATTCCGG-3′ (SEQ ID NO. 11)
PCR primer AP34	5′-ATACTCGGAATTGACGT-3′ (SEQ ID NO. 9)
PCR primer AP21	5′-TCCAATCGGTATCGCTC-3′ (SEQ ID NO. 17)
OXF6	5'-AATGTAGAGTTGACTGA-3' (SEQ ID NO. 12)
OXF7	5'-TTGATGCTGTTGATACG-3' (SEQ ID NO. 13)

The primer pair AP34 and AP21 (derived from *oxc* sequences between bp -59 to -41 and by 451 to 435, respectively), consistently amplifies a 500 bp segment of *oxc* from all *O. formigenes* strains and isolates tested. PCR application of whole fecal DNA with this genus-specific primer pair, in conjunction with Southern Blotting using genus and group specific probes, now provides a rapid diagnostic tool to detect and speciate *O. formigenes*. Time-consuming steps, *e.g.,* agarose-gel electrophoresis and Southern blot hybridizations, can be substituted with newer technologies such as microtiter-plate based colorimetric or fluorogenic assays (Jordan *et al.,*1996).

Polynucleotide primers contemplated by the subject invention also include any polynucleotide molecule comprising a nucleotide sequence capable of specifically priming

amplification of oxalyl-CoA decarboxylase or formyl-CoA transferase polynucleotide sequences disclosed herein. As used herein, reference to "substantial homology" or "substantially complementary" refers not only to polynucleotide primers of the subject invention having 100% homology with the nucleotide sequence of the target polynucleotide, or fragments thereof, but also to those sequences with sufficient homology to hybridize with and prime the amplification of a target polynucleotide. Preferably, the degree of homology will be equal to or about 100%. The skilled artisan, having the benefit of the disclosure contained herein, can readily prepare other primers of varying nucleotide length and sequence that can be used to amplify all or portions of the oxalyl-CoA decarboxylase and/or the formyl-CoA transferase gene.

The polynucleotide probes and primers of the subject invention can be chemically synthesized or prepared through recombinant means using standard methods and equipment. The polynucleotide probes and primers can be in either single- or double-stranded form. If the probe or primer is double-stranded, then single-stranded forms can be prepared from the double-stranded form. The polynucleotide probes and primers may be comprised of natural nucleotide bases or known analogs of the natural nucleotide bases. The probes and primers of the subject invention may also comprise nucleotides that have been modified to bind labeling moieties for detecting the probe or primer or amplified gene fragment.

The polynucleotide molecules of the subject invention can be labeled using methods that are known in the art. The polynucleotides may be radioactively labeled with an isotope such as ^{3}H, ^{35}S, ^{14}C, or ^{32}P. The polynucleotides can also be labeled with fluorophores, chemiluminescent compounds, or enzymes. For example, a polynucleotide molecule could be conjugated with fluorescein or rhodamine, or luciferin or luminol. Similarly, the polynucleotide molecule can be conjugated with an enzyme such as horseradish peroxidase or alkaline phosphatase. Polynucleotide molecules can also be detected by indirect means. For example, the polynucleotide may be conjugated with ligands, haptens, or antigenic determinants. The conjugated polynucleotide is then contacted with the ligand receptor, with an anti-ligand molecule that binds to the ligands, or with an antibody that binds to the

hapten/antigenic determinant, respectively. For example, the polynucleotide can be labeled with digoxygenin and detected with labeled anti-digoxygenin antibodies. The ligand receptor, anti-ligand molecule, or antibody may be directly labeled with a detectable signal system, such as a fluorophore, chemiluminescent molecule, radioisotope, or enzyme. Methods for preparing and detecting labeled moieties are known in the art.

The subject invention also concerns methods for quantitatively detecting oxalyl-CoA decarboxylase and formyl-CoA transferase nucleotide sequences and, thereby, determining the number of *Oxalobacter formigenes* in a sample. General methods and techniques for performing quantitative PCR are known in the art (Tarmuzzer *et al.*, 1996)

In one embodiment of the present detection method, samples to be tested for the presence of *Oxalobacter formigenes* are obtained from a person or animal, and DNA is isolated from the specimen using standard techniques known in the art. For example, cells can be lysed in an alkali solution, the nucleic acid extracted in phenol:chloroform, and then precipitated with ethanol. The DNA is then fragmented into various sizes using restriction endonuclease enzymes or other means known in the art. The DNA fragments are then electrophoretically separated by size on an agarose gel. In an alternative embodiment, the DNA fragments are subjected to PCR amplification using PCR primers of the present invention prior to gel electrophoresis in order to specifically amplify portions of the formyl-CoA transferase and oxalyl-CoA decarboxylase genes.

After the DNA fragments are separated on the gel, the size-fractionated DNA fragments are transferred to a membrane matrix, such as nitrocellulose, nylon, or polyvinylidene difluoride (PVDF), by Southern blotting. The DNA immobilized on the membrane matrix is single-stranded. Polynucleotide probes of the subject invention are then contacted with the membrane and allowed to hybridize with the DNA immobilized on the membrane. A probe of the present invention can be labeled with a detectable signal, such as a radioisotope, or the probe can be labeled with a hapten or antigen such as digoxigenin. The hybridization can be performed under conditions known in the art. After hybridization of the probe with the DNA fragments on the membrane, the membrane is washed to remove non-

hybridized probe. Standard wash conditions are known in the art, and the stringency and number of washes employed can vary.

The membrane is then tested or observed for the presence of hybridized probe. For example, if the hybridized probe was labeled with a hapten or antigen, then it can be detected using an antibody that binds to the conjugated hapten or antigen on the probe. The antibody can be directly labeled with a detectable fluorophore, chemiluminescent molecule, radioisotope, enzyme, or other signal generating system known in the art. Alternatively, the antibody can be detected using a secondary reagent that binds to the antibody, such as anti-immunoglobulin, protein A, protein G, and other antibody binding compositions known in the art. The secondary reagent can be labeled with a detectable fluorophore, chemiluminescent molecule, radioisotope, or enzyme. The presence of a detectable hybridization signal on the membrane indicates the presence of *Oxalobacter formigenes* in a test sample.

The subject invention also concerns a kit for the detection of *Oxalobacter formigenes* in a sample. A kit contemplated by the subject invention may include in one or more containers: polynucleotide probes, positive and negative control reagents, and reagents for detecting the probes. The kit may also include polynucleotide primers for performing PCR amplification of specific *Oxalobacter formigenes* genes. In a preferred embodiment, the polynucleotide probes and primers are specific for the oxalyl-CoA decarboxylase and formyl-CoA transferase genes of *O. formigenes*.

The subject invention also concerns a dipstick device comprising the enzymes of the subject invention and dyes and/or substrates immobilized on a carrier matrix. Any dye or substrate that yields a detectable product upon exposure to the reaction products that are produced by the enzymatic reaction of oxalate with oxalyl-CoA decarboxylase and formyl-CoA transferase as described herein is contemplated for use with the subject dipstick device. The carrier matrix of the assay device can be composed of any substance capable of being impregnated with the enzyme and dye components of the subject invention, as long as the matrix is substantially inert with respect to the analyte being assayed for. For example, the

carrier matrix may be composed of paper, nitrocellulose, PVDF, or plastic materials and the like.

Incorporation of the enzymes, dye and other components on the carrier matrix can be accomplished by any method such as dipping, spreading or spraying. A preferred method is impregnation of the carrier matrix material by dipping in a reagent solution and drying to remove solvent. Drying can be accomplished by any means which will not deleteriously affect the reagents incorporated, and typically is by means of an air drying oven.

The dipstick device of the subject invention is dipped in or contacted with a sample to be tested for the presence or amount of oxalate. Positive and negative controls can be used in conjunction with the dipstick device. An appropriate amount of time is allowed to pass and then the dipstick is assessed for a positive reaction by visual inspection. If oxalate is present in the sample then a detectable signal, usually in the form of a color, can be observed on the dipstick. Typically, the intensity of the color developed in a fixed time period is proportional to the concentration of oxalate present in the sample.

All publications cited herein are incorporated reference.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Determination of Level of Sensitivity of Enzyme Assay System

Samples containing oxalate at concentrations ranging from 0.004 mM to 0.00025 mM were prepared in 10 µl volumes. The samples were then assayed using the enzyme system of the subject invention in 96-well microtiter plates. Reagents were then added at the following concentrations: KH_2PO_4 (pH 6.7), 50 mM; $MgCl_2$, 5 mM; thiamine PPi (TPP), 2 mM; oxalyl-CoA, 0.375 mM; β-NAD, 1.0 mM; formate dehydrogenase, 0.25 IU; and oxalyl-CoA decarboxylase, 0.03 U. The reaction mixture was then incubated at 37°C for 2 minutes in order to permit the degradation of any residual formate that may be present in the sample

mixture. The reaction was then initiated by the addition of formyl-CoA transferase to the sample mixture. Changes in A_{340} were measured every 15 seconds at 37°C (Figures 1A-1E). Appropriate positive and negative controls were run simultaneously with the assay.

Example 2 - Detection of *Oxalobacter formigenes* in a Sample

Strains of *Oxalobacter formigenes* used in the following methods are listed in Table 1 below.

TABLE 1. Description of the *Oxalobacter formigenes* strains

Group Classification of *O. formigenes* strains[a]	Strain	Source of Isolate
Group I	OxB	Sheep rumen
	OxWR	Wild rat cecum
	SOx-4	Freshwater lake sediment
	SOx-6	Freshwater lake sediment
	POxC	Pig cecum
	HC-1	Human feces
Group II	BA-1	Human feces
	OxK	Human feces
	HOxBLS	Human feces
	HOxRW	Human feces
	OxCR	Lab rat cecum
	OxGP	Guinea pig cecum

[a] From Jensen and Allison (1994).

All *Oxalobacter formigenes* strains were grown in medium B containing 30 mM oxalate, as described in Allison *et al.* (1985). Human fecal samples (approximately 60 mg) were

inoculated anaerobically into vials containing 9 ml of media B, then sequentially transferred through 10^{-8} dilutions. Cultures were incubated at 37° C for 10 days and biochemically tested for the catabolic consumption of oxalate by $CaCl_2$ precipitation (50 µl media, 100 µl 1% $CaCl_2$, and 2.7 ml dH_20) and spectrophotometric analyses (600 nm).

Cultures (10-15 ml) of *O. formigenes* were centrifuged at 10,000 x g, the bacterial pellet was resuspended in 567 µl TE buffer (10 mM Tris-Cl, pH 7.5 plus 1 mM EDTA, pH 8.0), 30 µl 10% sodium dodecyl sulfate (SDS) and 3 µl of proteinase K (20 mg/ml), and the mixture incubated 5 hr at 37°C to ensure bacterial cell lysis. Nucleic acids were extracted from the lysates using phenol/chloroform/isoamylalcohol (25:24:1). Chromosomal DNA was precipitated from the aqueous phase by adding ½ volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol. DNA was recovered by centrifugation (12,000 x g), washed once with 70% ethanol, and the pellet resuspended in 15-20 µl H_2O. Bacterial DNA was also isolated directly from fresh human stool samples following lysis with chaotropic salt and guanidine thiocyanate, then binding to glass matrix (GlasPac, National Scientific Supply, San Rafael, CA) (Stacy-Phips *et al.*, 1995).

Bacterial DNA was digested with the restriction endonuclease Hind III (Life Technologies, Inc., Gaithersburg, MD). The restriction-enzyme generated fragments were size separated by gel electrophoresis through 0.5% agarose, stained with ethidium bromide (EtBr), illuminated with UV light, and photographed to document proper digestion. Digested DNA was then transferred from the agarose gels to positively-charged nylon membranes (Boehringer-Mannheim GmBH, Indianapolis, IN) by positive pressure blotting and UV cross-linking (Stratagene, LaJolla, CA). Hybridizations were carried out using internal sequence oligonucleotide probes. Oligonucleotides were synthesized in the University of Florida ICBR Oligonucleotide Synthesis Laboratory (Gainesville, FL) and have the sequences:

AP15 5′-GCACAATGCGACGACGA-3′ (SEQ ID NO. 6)

AP22 5′-GTAGTTCATCATTCCGG-3′ (SEQ ID NO. 11)

AP34 5′ATACTCGGAATTGACGT-3′ (SEQ ID NO. 9)

AP273 5′-TTCATGTCCAGTTCAATCGAACG-3′ (SEQ ID NO. 10).

Each oligonucleotide was end-labeled with digoxigenin in a reaction using terminal transferase. The digoxigenin-labeled oligonucleotide probes were hybridized to the immobilized DNA fragments and hybridization detected colorimetrically by enzyme-linked immunoassay (ELISA) using an anti-digoxigenin alkaline phosphatase conjugate according to the manufacturer's protocol provided with the GENIUS III detection system (Boehringer-Mannheim).

All PCRs were performed according to protocols described in Anderson *et al.* (1993). Briefly, 50 µl reactions contained 1.5 mM $MgCl_2$, 200 µM dNTP, 1.25 U Taq polymerase (GIBCO-BRL, Bethesda, MD), 1 µg template DNA and 1 µM each of a 5′ and 3′ primer. A preferred reaction profile proved to be 94°C for 5 min, then 45 cycles of 94°C for 1 min of denaturation, 55°C for 2 min of annealing and 72°C for 3 min of primer extension. PCR products were size separated by gel electrophoresis in 1.2% agarose containing EtBr and photographed in UV light. PCR primer AP15 (SEQ ID NO. 6) and primer AP22 (SEQ ID NO. 11) were used as primers.

Previous studies by Lung *et al.* (1994) showed that genomic DNA of *O. formigenes*, strain OxB, could be digested with the restriction enzyme Hind III and that a limited number of enzyme cleavage sites existed near or within the oxalyl-CoA decarboxylase (*oxc*) gene. A RFLP analysis of Hind III digested OxB genomic DNA using either probe AP15 (SEQ ID NO. 6), a probe homologous to an internal sequence of the *oxc* gene, probe AP34 (SEQ ID NO. 9), a probe homologous to a 5′ -end sequence of the *oxc* gene but separated from the probe AP15 (SEQ ID NO. 6) sequence by a Hind III site, or probe AP273 (SEQ ID NO. 10), a probe homologous to an internal sequence of the formyl-CoA transferase (*frc*) gene, is shown in Figure 4. Using probe AP15 (SEQ ID NO. 6), a fragment of approximately 7 kb containing a portion of the *oxc* gene was detected, while fragments of approximately 3 kb were detected using either probe AP34 (SEQ ID NO. 9) or probe AP273 (SEQ ID NO. 10). The 3 kb fragment identified by probe AP34 (SEQ ID NO. 9) is distinct from the 3 kb fragment detected by probe AP273 (SEQ ID NO. 10).

As shown in Figure 5, the oxalyl-CoA decarboxylase and formyl-CoA transferase genes were consistently detected in samples containing as little as 0.06 to 0.20 μg of *O. formigenes*, strain OxB, DNA or approximately 0.20 to 0.40 μg of *O. formigenes* DNA from other group I strains, such as HC-1. The 23-bp probe AP273 (SEQ ID NO. 10) can detect the *frc* gene in DNA samples containing only one-fourth the amount of DNA required for the 13 bp probe AP15 (SEQ ID NO. 6) to detect the *oxc* gene (Fig. 5, upper panel). These probes are highly specific for *O. formigenes* since they fail to bind to other bacterial DNA, including *Escherichia coli, Alcaligenes oxalaticus*, and fecal bacteroides.

Protein, lipid and genetic studies of several isolates of *O. formigenes* have provided the basis for dividing this genus into two major subgroupings (Jensen *et al.*, 1994). When RFLP analyses were performed on genomic DNA isolated from various *Oxalobacter formigenes* strains, probes AP15 (SEQ ID NO. 6) and AP273 (SEQ ID NO. 10) were able to distinguish group I strains from group II strains on the Southern blot hybridizations (Fig. 6). All strains of *O. formigenes* belonging to group I (to which OxB is assigned) hybridized with both probe AP15 (SEQ ID NO. 6) and probe AP273 (SEQ ID NO. 10). Due to a characteristic slow growth of strain HC-1, only faint bands appeared in this experiment. In contrast, none of the *O. formigenes* strains assigned to group II hybridized with probe AP273 (SEQ ID NO. 10) and only BA-1 hybridized with probe AP15 (SEQ ID NO. 6). These data indicate a highly conserved homology of *oxc* and *frc* within group I strains and a less conserved homology within group II strains.

To increase the sensitivity of detecting *O. formigenes*, PCR was used to amplify that portion of *oxc* which by RFLP appeared to differentiate the group I and group II strains. Using primer AP15 (SEQ ID NO. 6) and primer AP22 (SEQ ID NO. 11) as PCR primers to amplify a DNA segment in the carboxy-terminal region of *oxc*, strains assigned to group I (*i.e.*, OxB, HC-1, OxWR, POxC, SOx-4 and SOx-6) exhibited a common band at 452 bp (Fig. 7). In contrast, the other six strains, all belonging to group II, showed variable amplification patterns, but all showed a dominant PCR band of approximately 630 bp, with a weaker 452 bp band. Sequence analysis of this 630 bp band from strain OxK has revealed

the presence of the 452 bp sequence present in the 630 bp PCR product. Close analysis of the group II strains suggest that their PCR amplification profiles are highly reproducible, suggesting group II strains may fall into three (sub)groupings: HOxBLS and HOxRW (subgroup 1), OxCR and OxGP (subgroup 2), and BA-1 and OxK (subgroup 3).

The use of PCR-based detection of the *oxc* gene to identify *O. formigenes* in clinical specimens was examined by comparing PCR and biochemical methods of detection. Specimen 1, known to be positive for *O. formigenes*, gave ambiguous results in biochemical testing for oxalate depletion, but exhibited the presence of the 450 bp PCR product indicative of an *O. formigenes* group I strain. Specimen 2, known to be negative for *O. formigenes*, proved negative using both PCR-based and biochemical testing. Specimen 3, known to be positive for *O. formigenes*, showed depletion of oxalate in all dilutions and revealed a PCR pattern suggestive of an *O. formigenes* group II strain. PCR amplification was not observed in the original culture or the first dilution due to the presence of inhibitors of PCR *e.g.*, bile salts, bilirubin, etc.) which copurify with DNA.

To circumvent the inhibition of the PCR by factors co-purifying with the bacterial DNA, DNA isolation was performed by lysing fresh stool samples with guanidine thiocyanate followed by adsorption to and elution from glass matrices. Using this method, PCR-based detection of *O. formigenes* can be performed using fecal DNA diluted only 1:25 to 1:50 to eliminate PCR inhibitors. Sensitivity experiments using different stool samples spiked with strains OxB or OxK in the range of 10^1 to 10^7 cfu per 0.1 g of sample showed that as few as 10^2 to 10^3 cfu of *O. formigenes* per 0.1 g sample could be detected (Figure 8). Again, PCR-based analyses of DNA isolated directly from a stool sample known to be positive for *O. formigenes* by culture methods, showed amplification patterns indicative of a group II strain (Figure 8, lanes F & G).

Example 3 - Detection and Classification of *Oxalobacter formigenes*

Bacterial Strains

O. formigenes strains used included *OxB* (isolated from sheep rumen) and *HC1, OxK, BA1, HOxBLS, HOxRW, HOxRA, HOxCC13, and HOxHM18* (isolated from human feces). In addition, several new purified cultures, including *HOxUK5, HOxUK88, HOxUK90,* and *HOxHS* (grown from human feces), were also used. All strains and isolates were grown in media B containing 30 mM potassium oxalate, as described elsewhere (Allison *et al.,* 1985), and maintained under strict anaerobic conditions until used.

Preparation of Genomic DNA from *O. formigenes* Cultures

Fifteen ml cultures of *O. formigenes* were centrifuged at 10,000 x g, the bacterial pellet resuspended in 567 µl of TE buffer (10 mM Tris-HC1, pH 7.5, plus 1 mM EDTA, pH 8.0), 30 µl of 10% sodium dodecyl sulfate plus 3 µl of proteinase K (20 mg/ml), and this mixture incubated for 5 hours at 37°C to ensure bacterial cell lysis. Nucleic acids were extracted from the lysates with phenol:chloroform:isoamyl alcohol (25:24:1). Chromosomal DNA was precipitated by adding ½ volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol. DNA was recovered by centrifugation (12,000 x g) and washed once in 70% ethanol. The final DNA precipitation was resuspended in 20 µl H_2O.

Sequence Analysis of the *oxc* Genes

The primer pair,

5′-ATACTCGGAATTGACGT-3′ (a 5′-primer designated AP34) (SEQ ID NO. 9)

and

5′-TCCAATCGGTATCGCTC-3′ (a 3′-primer designated AP21) (SEQ ID NO. 17)

homologous to sequences within the 5′-end of the *oxc* gene present in strain *OxB* (Lung *et al.,* 1994), was used to amplify a 500 bp DNA fragment from genomic DNA isolated from each of twelve human *O. formigenes* strains. Amplifications were performed in 50 µl PCR reactions containing 1.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphate, 1.25 U of Taq

polymerase (Gibco-BRL, Bethesda, MD), 1 μg of genomic DNA and 1 μM each of 5′- and 3′-primer. PCR were carried out for 35 cycles and included an initial 5 minute denaturation step at 94°C, 1 minute annealing (with a temperature stepdown from 60°C to 55°C), 1 minute extension at 72°C and a final 8 minute extension at 72°C. The PCR products were size fractionated by electrophoreses through 1.2% agarose gels containing ethidium bromide for visualization of the bands in UV light. Each 500 bp PCR product was cloned into the TA cloning system, pCR-2.1 (Invitrogen, Inc., San Diego, CA). Competent DH5∝ *E. coli* bacteria were transfected with the recombinant plasmid and transformed bacteria selected on LB agar plates containing 10 μl/ml of ampicillin and 20 mg/ml of X-Gal. DNA from appropriate clones was isolated, checked for the presence of an insert of correct size by digestion with the restriction enzyme, Eco RI. Inserts of recombinant plasmids were sequenced using M13-forward and M13-reverse primers.

Clinical Samples

Fecal samples of 100 generally healthy children of either sex ranging in age from 0 to 12 years were examined for the presence of *O. formigenes*. All fecal samples were collected in Dzerzhinsk, a city in the Donetsk region of the Ukraine. This particular population was selected due to the fact that these children have had limited use of antibiotics, that might influence bacterial colonization of the intestinal tract, in treatment of childhood diseases. Approximately 25 mg sample of fresh stool (within 3-4 hours of collection), was inoculated into vials containing 10 ml of anaerobically sealed media B supplemented to 30 mM with potassium-oxalate. The vials were analyzed at the University of Florida, Gainesville, FL. After incubation at 37°C for one week, the loss of oxalate from each fecal culture was determined using a calcium-chloride precipitation method in which 50 μl culture media is mixed with 100μl 0.1% $CaCl_2$ plus 3.0 ml dH_2O and the absorbance of each mixture determined spectrophotometrically (600nm). The calcium precipitation test for loss of oxalate has been repeatedly verified as reliable by other methods (*e.g.*, gas chromatography and butyl esters) for detection of oxalate. Typically, cultures not showing catabolism of oxalate

generally have O.D. readings of about 0.1, whereas cultures with oxalate degradation have O.D. readings less than about 0.02.

PCR-based Detection and Identification of *O. formigenes*

DNA was isolated from individual fecal cultures by the method of Phipps *et al.* (Stacy-Phipps *et al.*, 1995) using guanidine thiocyanate as a chaotropic agent and glass-matrix for DNA binding. One µl of each DNA sample was used as template in a 50 µl PCR reaction as described above. The amplified PCR products were size separated by electrophoresis through 1.2% agarose gels containing ethidium bromide and visualized with UV light. Each reaction was controlled using a reaction containing all the components of the PCR with the exception of template DNA.

Southern Blot Analysis

Southern blots were carried out as previously detailed in Example 2. Briefly, the size separated PCR products were transferred to positively charged nylon membranes (Boehringer Mannheim GmBH, Indianapolis, IN) by positive pressure blotting and UV-crosslinking. The *oxc* derived genus specific (AP286), group I specific (HS-2) and group II specific (AP307) oligonucleotides were synthesized in the University of Florida ICBR DNA Synthesis Laboratory (University of Florida, Gainesville, FL) and end-labeled with digoxigenin in a reaction using terminal transferase. The digoxigenin labeled oligonucleotides were hybridized to the immobilized PCR products under conditions of high stringency (5X SSC and 68°C). Hybridization was detected colorimetrically by enzyme-linked immunosorbent assay (ELISA) with an anti-digoxigenin alkaline phosphatase conjugate according to the manufacturer's protocol provided with the GENIUS III kit (Boehringer Mannheim GmBH).

Generation of Genus-specific and Group-specific Probes

Preliminary studies looking at the efficacy of various oligonucleotide pairs to amplify portions of the *oxc* gene present in various *O. formigenes* strains revealed that the PCR

primer pair AP34 (5′-primer)/Ap21 (3′-primer) amplified a 500 bp DNA fragment in both group I and group II strains. To determine the degree of sequence homology within the 5′-end of the *oxc* gene between various strains of *O. formigenes*, genomic DNA was prepared from 5 group I and 7 group II strains isolated from human fecal samples for use as template in PCR with AP34 and AP21. Each PCR amplified an expected 500 bp product that was subsequently cloned into the pCR-2.1 vector system and sequenced. A comparison of the 5′-end sequences of the *oxc* gene from these 12 human isolates with the *OxB* gene is shown in part in Figures 9A-9B. The 5′-end of the *oxc* gene appears to be relatively conserved for a bacterial gene, with most bp changes occurring in the wobble base such that the codon translation is not altered. Nevertheless, there were enough sequence differences to demarcate group I strains from group II strains, thus permitting selection of regions that are conserved within strains of a specific group, but differ significantly from strains of the other group. Based on these conserved regions, genus-specific oligonucleotide probes (for example, probe AP286, homologous to the region between bp 13 and 43 of the open-reading frame), as well as group I-specific (for example, probe HS2, homologous to the region between bp 197 and 214 of the open-reading frame) and group II-specific (for example, probe AP307, homologous to the region between bp 133 and 150 of the open-reading frame) probes were prepared.

Specificity of the Genus-specific and Group-specific Oligonucleotide Probes

The specificity of probes AP286, AP307, and HS2 in detecting and classifying *O. formigenes* was examined using genomic DNA prepared from a number of known strains and isolates. PCR amplifications with the genus-specific primer pair AP34 and AP21 resulted in the 500 bp amplification product in all cultures tested (Figure 10A, top panel). On Southern blotting, this 500 bp fragment hybridized with a genus-specific probe, AP286 (Figure 10B, bottom panel).

In a separate experiment, the amplified 500 bp PCR product was hybridized with either the group I-specific probe, HS2, (Figure 11B, middle panel) or the group II-specific

probe, AP307, (Figure 11C, bottom panel). Results clearly show a group specificity in the binding of these group-specific probes and their ability to identify subgroups of *O. formigenes*.

Application of a PCR-based Detection System for *O. formigenes*

In a double-blinded study, 100 fecal samples were collected from children ranging in age from newborn to 12 years and tested for the presence of *O. formigenes* using both an oxalate degradation system and our PCR-based assay system. The aim of this study was to determine the age at which children become naturally colonized with this intestinal anaerobic bacterium. Of the 100 fecal samples examined, 72 samples tested positive for *O. formigenes* by PCR, 59 of which also exhibited oxalate degradation in an oxalate degradation assay. Interestingly, of the 72 positive samples, 68 were group II strains while only 4 were group I strains. All fecal cultures exhibiting degradation of oxalate tested positive for *O. formigenes* by PCR. Although there were 13 cultures that failed to degrade oxalate that proved positive for *O. formigenes* by PCR, the majority of the samples that failed to degrade oxalate also failed to exhibit amplification of a product in the PCR-reaction. These data show that the PCR-based assay is probably more sensitive than the biochemical (calcium chloride precipitation) test, yet highly specific.

When the data were unblinded, a clear pattern for the natural colonization of children became evident. *O. formigenes* could not be detected in infants less than 6-9 months of age. *O. formigenes* began appearing in the intestinal tracts of children around 1 year of age, and by 3-4 years of age, all children showed signs of being colonized. Although the sample size is small, the number of children colonized with *O. formigenes* declined between 8-12 years of age, reaching the colonization frequency of 70-80% estimated for adult populations (Doane *et al.*, 1989, Kleinschmidt *et al.*, 1993, Allison *et al.*, 1986, and Goldkin *et al.*, 1985).

Example 4 - Quantitative DNA-based detection and identification of *Oxalobacter formigenes*

Because the original PCR primer pair AP34/AP21 was based on the nucleotide sequence encoding oxalyl-CoA decarboxylase in strain *OxB* (isolated from sheep), a PCR primer pair based on conserved sequences found in human strains was used. This primer pair, OXF6 and OXF7, amplifies a 416 bp product. The nucleotide sequences of OXF6 and OXF7 are shown below:

OXF6 5'-AATGTAGAGTTGACTGA-3' (SEQ ID NO. 12)
OXF7 5'-TTGATGCTGTTGATACG-3' (SEQ ID NO. 13)

To develop a competitive DNA template for use as an internal control for QC-PCR, a 227 bp fragment of the oxalyl-CoA decarboxylase gene *(oxc)* flanked by sequences homologous for the OXF6/OXF7 primer pair and containing the genus-specific, group I-specific and group II-specific probe sites was generated. To accomplish this, a PCR reaction was performed using the OXF6 5'-primer plus a modified OXF7 3'-primer. The modified 3'-primer consisted of two portions: a 5'-end which contained the 3'-primer sequence within the *oxc* gene plus a 3'-end which annealed at a site located approximately 200 bp downstream of the 5'-primer site. The PCR using the primer pair OXF6/modified-OXF7 amplified the 210 bp segment and synthesized the 17 bp OXF7 primer site at the 3'-end. This PCR fragment was purified and ligated into pCR-2.1 (Invitrogen, Inc., San Diego, CA). A recombinant pCR-2.1 plasmid with the proper insert (confirmed by sequencing) was selected for use as the internal competitive template.

Quantitative PCR (QC-PCR) were performed as described below. Competitive template diluted from 1×10^{10} to 1×10^{2} copies/PCR were used to establish standard curves. Experimental PCRs were established containing both experimental *O. formigenes* DNA and dilutions of the synthetic template. The PCR products were size separated by electrophoresis through 1.5% agarose gels visualized with UV light and photographed (Figures 12A-12C). Photographs were scanned for relative band intensities, normalized for differences in

molecular mass, and plotted against the log of the copy number of synthetic template added per reaction.

Quantitation of the number of *oxc* genes, thereby the number of bacteria in a sample, revealed the accuracy of this QC-PCR detection system. Assuming the genome of *O. formigenes* is similar in size to that of *E. coli* (4.7×10^3 Kb), then 1 μg of genomic DNA would contain 1.8×10^8 molecules (or gene copies). Genomic DNA prepared from *O. formigenes OxB* had a concentration by spectrophotometric analysis of 1.126 μg DNA/μl or 2×10^8 molecules/μl. Two dilutions, 10^4 (20,000 molecules) and 10^6 (200 molecules) of this DNA were used as template in the QC-PCR with dilutions of competitive template ranging from 50 to 250,000 molecules. As shown in Figures 12D-12E, the Log equivalence revealed that the number of molecules of *O. formigenes OxB* in the reaction were estimated between 19,900-25,100 and 126-158.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

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